The rate equations of the association reactions were calculated from these data and found to be:

(t-Chloride) $k = 10^{11.0 \pm 1.0} e^{-(28,800 \pm 2400)/RT}$

(t-Bromide) $k = 10^{10.2 \pm 0.7} e^{-(22,500 \pm 1900)/RT}$

The units are cc. moles^{-1} sec.$^{-1}$.

The isomerization of t-butyl chloride to isobutyl chloride at 270° was investigated, and found to be less than 7.8% at equilibrium.

The statistical mechanical explanation of slow reaction rates in the form developed by Eyring was applied, and reasonable agreement was found with the theory.

CAMBRIDGE, MASS. RECEIVED NOVEMBER 2, 1936

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY AND THE DEPARTMENT OF HYGIENE AND BACTERIOLOGY, UNIVERSITY OF CHICAGO]

Some Chemical Properties of an Essential Growth Factor for Pathogenic Bacteria

BY FELIX SAUNDERS, I. I. FINKLE, LEON STERNFELD AND STEWART A. KOSER

Many bacteria which develop quite readily in a meat-infusion culture-medium are unable to grow in a simple synthetic medium composed of several amino acids, dextrose, and inorganic salts. This observation is particularly true of many of the pathogenic types and has given rise to considerable speculation concerning the nature of the substances in meat infusions which appear to be necessary for development. Several attempts have been made to fractionate meat infusions or other similar mediums in the hope of gaining an insight into the nature of the factors essential for growth of the more exacting bacteria.[1] The same method of study has also been applied to yeasts.[2]

In previous work of our own[3] it was found that a growth-stimulating fraction could be removed from veal infusion by adsorption on charcoal and subsequent elution with hot ethanol or acetone. When this fraction was added to a simple synthetic medium[4] it rendered the medium suitable for the growth of some, though not all, of the more exacting pathogenic bacteria. In certain instances very small amounts of this added factor were sufficient to permit development in a synthetic medium in which the organisms ordinarily refused to grow. The results of a representative experiment with three microörganisms belonging to different biological groups are given in Table I.

The luxuriance of growth, varying from very light to heavy turbidity, is shown by the number of + signs. Absence of visible turbidity is shown by 0. An occasional doubtful reading is recorded ?. Readings were also made after seven and fourteen days, but have been omitted from the table as in most instances they showed no change.

The strain of *C. diphtheriae* used was a culture of "Park 8," that of the Sonne dysentery bacillus was no. 268 of the British Type Collection, and the strain of *Staph. albus* was from our laboratory stock collection.

Here it is seen that two of the three microorganisms refused to develop in the synthetic medium alone. The third organism, the so-called Sonne type of dysentery bacillus, grew very sparsely in the synthetic medium. When the fraction from veal infusion was added all of the organisms developed very well and produced a heavy growth in a few days. Relatively small amounts of this added material were effective and it is evident that something quite essential for multiplication has been added.

Is the added material a foodstuff or something in the nature of an accessory growth factor? It is obvious that the fraction from veal infusion must consist of a mixture of compounds and one would expect some food materials to be carried over along with any accessory factor which might be present. The results with the inorganic salt solution shown in Table I indicate that only a negligible amount of actual food material was contained

(1) (a) Thjötta and Avery, *J. Exptl. Med.*, **34**, 97, 455 (1921); (b) Mueller, *J. Bact.*, **7**, 309 (1922); (c) Mueller, *et al.*, *ibid.*, **25**, 509 (1933); **30**, 513 (1935); (d) Whitehead, *Biochem. J.*, **17**, 742 (1923); **18**, 829 (1924); (e) Knight and Fildes, *Brit. J. Exptl. Path.*, **14**, 112 (1933); (f) Sahyun *et al.*, *J. Infectious Diseases*, **58**, 28 (1936).

(2) See especially Williams, *et al.*, THIS JOURNAL, **55**, 2912 (1933); *Proc. Soc. Exptl. Biol. Med.*, **32**, 473 (1934); Miller, Eastcott and Maconachie, THIS JOURNAL, **55**, 1502 (1933); Kögl, *Ber.*, **68**, 16 (1928).

(3) Koser and Saunders, *J. Infectious Diseases*, **56**, 305 (1935).

(4) The synthetic medium consisted of 1.4 g. Na_2HPO_4, 1.0 g. KH_2PO_4, 2.0 g. NaCl, 0.1 g. $MgSO_4$, 3.0 g. asparagine, 0.1 g. cystine, 0.2 g. tryptophane, and 2.0 g. dextrose in 1 liter of distilled water. When an inorganic salt solution was desired for control purposes the asparagine, cystine, tryptophane and dextrose were omitted.

TABLE I

EFFECT OF ADDING A FRACTION FROM VEAL INFUSION TO A SYNTHETIC MEDIUM

Microörganisms used	Amount of added fraction,[a] cc.	Development in synthetic medium, days 1	2	4	Development in inorganic salt solution, days 1	2	4
Corynebacterium diphtheriae	None	0	0	0	0	0	0
	0.1	+	++	+++	0	0	0
	.01	0	+	++	0	0	0
	.001	0	0	+			
	.0001	0	0	0			
Shigella dysenteriae Sonne type	None	0	?	+	0	0	0
	0.1	+++	+++	+++	+	+	+
	.01	+++	+++	+++	0	?	?
	.001	+++	+++	+++	0	0	0
	.0001	+++	+++	+++	0	0	0
	.00001	+	+	+	0	0	0
Staphylococcus albus	None	0	0	0	0	0	0
	0.1	+	++	+++	?	+	++
	.01	0	+	++	0	0	0
	.001	0	0	0	0	0	0
	.0001	0	0	0	0	0	0

[a] The amount of solution of growth factor (from veal infusion) added to each 5-cc. tube of either synthetic medium or inorganic salt solution. The total solids in each cc. of the preparation of growth factor was 3 mg.

in the fraction from veal infusion. Since the inorganic salt solution supplied no food material which could be used by the bacteria for growth, any development following addition of the fraction from veal infusion must have resulted from food material carried over in this fraction. When large amounts such as 0.1 cc. of this preparation were added to the inorganic salt mixture, a light growth of two of the cultures resulted but in no case was the development nearly as rapid or as luxuriant as when a similar amount was added to the synthetic medium. Nor was growth stimulated by such small amounts of the fraction as was evident in the case of the synthetic medium. The preparation alone evidently supplied only traces of food material but when added to a synthetic medium containing dextrose and a few amino acids, very small amounts were sufficient to serve as an "activator" and the amino acids and dextrose were then utilized with resultant good development of the cultures.

Later work[5] showed that a similar preparation could be obtained from a considerable number of animal and plant sources. Of over thirty such sources tested, calf spleen, calf liver, and yeast appeared to be the richest. The evidence accumulated so far indicates that the growth factor is widely distributed in living tissue whether of plant or animal origin, and is probably a single chemical entity. Additional study of this apparently essential growth factor seemed desirable and attempts were made to obtain the active substance in pure form. Although this objective has not been reached up to the present time, some progress has been made in the fractionation, and with the partially purified preparations obtained it has been possible to study some of the chemical properties of the active principle.

(5) Koser, Saunders, Finkle and Spoelstra, *J. Infectious Diseases*, **58** 12 (1936).

Experimental

Preparation of Crude Growth Factor.—For the preparation of the fractions used in the following studies usually beef spleen or liver were used.[6] The method for concentrating growth factor has undergone several modifications. The process given below is the one in use at present. One hundred pounds (45 kg.) of beef spleen or liver are ground and covered with 250 liters of distilled water. After standing overnight or longer in the refrigerator the mixture is heated to boiling and boiled for five minutes. It is filtered while hot and the residue is washed with 50 liters of boiling water. The *hot* filtrates are combined and a *hot* saturated solution of lead acetate is added until no more precipitate is formed. It is important that *both* solutions be hot, otherwise the next filtration is likely to be very slow. Fuller's earth and supercel (200 g. of each) are stirred in thoroughly. The lead precipitate and the earths are filtered off. Charcoal is suspended in the filtrate (about 1 g. for every liter of filtrate added in small portions) and stirred intermittently for ten minutes or more. The charcoal is filtered off under suction and washed until free from lead.

The wet charcoal is placed in a large continuous extractor and extracted with alcohol for two weeks or more if the

(6) We are indebted to Dr. David Klein of The Wilson Laboratories for generously supplying us with tissue and carrying out some of the preliminary steps of the purification.

alcohol still shows considerable color. The alcohol is changed every other day and the several portions of alcohol are combined. Every day 100 cc. of water is poured into the extractor through the condenser. When most of the color has been removed from the charcoal the extraction is discontinued and the charcoal discarded. The alcohol is removed from the combined extracts under reduced pressure. The residue is taken up in a minimum of water and any insoluble material is filtered off. The filtrate is again taken to dryness in a flask with a ground glass joint which can be fitted to a condenser.

The residue is extracted at least three times with 200-cc. portions of boiling absolute methanol. The methanol extracts are decanted or filtered from the residue in the flask. To the combined methanol extracts, 5 volumes of absolute ether are added. After standing a few minutes a gummy precipitate settles out leaving a clear supernatant fluid which can be decanted. The methanol–ether mixture is removed under reduced pressure. The residue is taken up in 100 cc. of water or more if necessary and any insoluble material is filtered off.

Fifty cc. of a 10% solution of chloroplatinic acid is added to the filtrate which is placed in the refrigerator and allowed to stand for a week or more. The precipitate which has formed is filtered off. The filtrate is allowed to stand overnight. If no more precipitate forms hydrogen sulfide is passed into the solution to remove the platinum. If a precipitate does form the filtrate is allowed to stand for a few days more and the second precipitate is removed.

After the platinum sulfide has been filtered off the filtrate is evaporated to dryness under reduced pressure. The residue is taken up in water and any insoluble material filtered off. The filtrate constitutes the crude material on which most of the tests described in this paper were performed. The solutions are straw colored or brownish and have a characteristic meat-extract odor. This odor is the same regardless of the source from which the material was prepared.

Method of Assay.—Four different organisms, *Staphylococcus albus*, *Corynebacterium diphtheriae* (Park 8), *Shigella dysenteriae* (Shiga), and *Brucella abortus*, were used for assay. These organisms show a wide range of sensitivity toward the growth factor but could always be arranged in the same order of sensitivity regardless of the source of the growth factor or the treatment to which it had been subjected. Throughout the experiments care was taken to avoid carrying over a heavy inoculum when testing for the presence of growth-promoting factor. Light suspensions of each of the four organisms were first made in 5 cc. of a buffered salt solution by transferring a small bit of growth from a twenty-four- or forty-eight-hour agar slant of the culture. These suspensions showed no evident turbidity. After thorough distribution, 0.1 cc. of the suspension was then pipetted aseptically into each tube of synthetic medium containing the added extract and also into controls of synthetic medium alone and of veal infusion broth. All tests were incubated at 37°. Throughout the work the purity of the cultures developing in the various tests was often checked by gram stains together with whatever other study appeared desirable.

Solubility in Organic Solvents.—A solution of growth factor was shaken with three separate portions of ether. The ether fractions were combined and the ether removed under diminished pressure. The slight residue was taken up in water and assayed but no activity could be detected. Similar results were obtained from the same procedures using benzene and chloroform. The higher alcohols appear to dissolve some growth factor but the growth factor is probably held in the small amount of water which the alcohols dissolve. The growth factor is soluble in absolute methanol and absolute ethanol but not as readily as in water. However, it is still too soluble in these alcohols to permit recrystallization from them. In one experiment a concentrated solution of growth factor in ethanol was prepared and chilled with solid carbon dioxide until it became sirupy but no solid was thrown out. Of course the growth factor is readily soluble in any aqueous mixture of the alcohols since it is extremely soluble in water.

Solubility in Phenol.—A growth-factor preparation was evaporated to dryness. Phenol was added and warmed until it liquefied. All of the material appeared to go into solution. The flask was washed out with another portion of liquefied phenol and the combined portions of phenol were filtered through a steam funnel. The phenol was removed from the filtrate by steam distillation. The liquid remaining in the flask after removing the phenol was evaporated to dryness under diminished pressure, taken up in the original amount of water and assayed. Little or none of the activity had been destroyed by the treatment described.

Effect of Oxidants on Growth-Factor Preparations.—Enough 30% hydrogen peroxide was added to 50 cc. of a solution of growth factor to give a final concentration of 3% and the solution heated on the steam-bath for forty-five minutes. The solution was evaporated to dryness under reduced pressure and the residue taken up in 50 cc. of water. Another sample of growth factor in aqueous solution was aerated by passing a vigorous stream of air through a flask containing the solution for twenty hours. Enough water was then added to restore the original volume. Assay showed that in neither of these samples was the activity appreciably diminished. Heating a sample of growth factor on the steam-bath under reflux for thirty-six hours or autoclaving for fifteen minutes at 1 atm. extra pressure had no detectable effect on the activity.

Action of Bromine on Growth Factor.—Bromine was added drop by drop to a solution of growth factor with vigorous shaking until the color persisted. The solution was allowed to stand overnight in a refrigerator. Excess bromine was removed by aeration and a gummy precipitate which had formed was filtered off. Silver acetate was added to remove bromide ions and the silver bromide removed by filtration. Hydrogen sulfide was passed into the solution and the silver sulfide formed was filtered off. The filtrate was concentrated under reduced pressure to dryness and then taken up in enough water to make up the original volume of the solution. The activity had been reduced by about one-fourth. In another experiment the bromine was added in solution in chloroform. The results were essentially the same as described above. When a large excess of liquid bromine is added to a dried growth-factor preparation and heated on the steam-bath the activity is destroyed completely.

Action of Ammoniacal Silver on Growth Factor.—Ammoniacal silver was added to a growth-factor preparation

and the solution was warmed on the steam-bath for ten minutes. The calculated amount of hydrochloric acid was added and the silver chloride removed. There was no loss of activity. None of the preparations tested reduced Fehling's solution.

Action of Acetic Anhydride on Growth Factor.—A solution of growth factor was evaporated to complete dryness *in vacuo*. Seventy-five grams of acetic anhydride and 5 g. of fused sodium acetate were added. The mixture was heated on the steam-bath under reflux for twenty minutes and then poured into 500 cc. of iced water. A gummy precipitate formed which was removed by filtration. The filtrate was diluted to 2 liters and treated with 15 g. of charcoal in two steps to adsorb the growth factor. The charcoal was filtered off and extracted in a continuous extractor for twenty-four hours. The alcohol was removed under reduced pressure and the residue taken up in water. Assay showed that there had been only a slight loss of activity.

Action of Nitrous Acid on Growth Factor.—Fifty cc. of a preparation was diluted to 2 liters and 10 g. of sodium nitrite added. The solution was cooled to 10° and 25 cc. of glacial acetic acid added. The solution was stirred for thirty minutes and then allowed to stand overnight in the refrigerator. The growth factor was adsorbed on 8 g. of charcoal and eluted with alcohol. There was only a slight loss of activity.

Qualitative Elementary Tests.—Qualitative tests for nitrogen were run on several different samples. In every case the test was positive. Tests for sulfur run on the same samples were always negative. Since the preparations were impure no significance can be attached to the nitrogen tests but the growth factor is probably free from sulfur.

Discussion

All the experiments described above have been repeated and confirmed several times. They have also been repeated in many cases using growth factor from different sources. All the evidence at hand indicates that the growth factor is the same regardless of source and that it is a widely distributed if not universal constituent of living tissue. Many microörganisms are unable to utilize sources of energy unless this accessory substance is present. Others which are capable of developing in a synthetic medium apparently can either get along without it or can synthesize the small amount of growth factor that they need. For example, veal infusion from which the growth factor has been removed by adsorption on charcoal will still support the growth of airborne saprophytes and molds but will not permit the growth of certain pathogens. However, after saprophytes have been allowed to grow in the infusion which has been treated with charcoal the presence of growth factor can be demonstrated by the technique described above. Furthermore molds grown on Czapek-Dox medium will synthesize growth factor.

Because of the wide distribution of growth factor and the organisms capable of synthesizing it, it would be difficult or impossible to produce a diet deficient in this factor for higher forms, especially as the intestinal tract contains organisms capable of synthesizing this accessory. Growth factor requirements can be studied only under rigidly controlled conditions such as those prevailing in bacterial cultures in a synthetic medium. Perhaps organisms in the intestinal tract play an essential part in supplying this factor and are a part of a true symbiosis.

There is considerable variation among bacteria regarding their growth-factor requirements. Some require only a few micrograms of growth factor for each liter of medium in order to produce abundant growth while others grow much less vigorously even when the amount of growth factor has been increased as much as a hundred fold. Of course, many other factors such as amino acids, sugars, etc., may be involved. Cultures of *C. diphtheriae* have been grown on synthetic medium plus growth factor by transferring directly from one synthetic culture to another without any intermediate culturing on infusion. This culturing has been carried on for over fifty successive transfers.

In spite of its biological reactivity, growth factor is comparatively unreactive chemically. Although it has the stability generally associated with hydrocarbons it is extremely water soluble. Dried growth-factor preparation dissolves in water almost instantaneously but is not hygroscopic. It probably is not inorganic since it is destroyed by both wet and dry ashing. At present we feel that we have not enough positive chemical evidence at hand to warrant drawing any conclusions regarding its chemical constitution.

Acknowledgment.—This work has been aided by a grant from the Committee on Scientific Research of the American Medical Association. It is a pleasure to be able to thank the Committee for their generous support.

Summary

1. Many plant and animal tissues contain a substance which is essential for the growth of certain pathogenic bacteria. This substance has been purified partially and the chemical properties of the impure preparations studied.

2. Growth factor is soluble in water, metha-

nol, ethanol, and phenol, but it is insoluble in the higher alcohols, ether, benzene, and chloroform.

3. The activity is not appreciably affected by autoclaving, aeration, boiling, or by oxidizing agents such as 3% hydrogen peroxide, or ammoniacal silver, and is only slightly affected by bromine in the cold.

4. Treatment with acetic anhydride caused only slight loss of activity.

5. The growth factor probably does not contain sulfur.

6. It is not inorganic since it is destroyed by both wet and dry ashing.

CHICAGO, ILL. RECEIVED NOVEMBER 14, 1936

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CHICAGO]

Studies in Proteins. V. A Crystalline Globulin from the Paradise Nut, Lecythis Zabucayo

BY BIRGIT VENNESLAND, MARY B. BLAUCH AND FELIX SAUNDERS

Introduction

The Paradise nut, *Lecythis zabucayo*, is a rugose, fusiform nut resembling the Brazil nut in size and taste. It is grown in Brazil and Guiana where it is known as the sapucaya or monkey-pot nut. The natives consider the flavor of the Paradise nut to be superior to that of the Brazil nut, consequently few of them are exported but they can be obtained occasionally on the Chicago market.

Since the Brazil nut yields a crystalline globulin, excelsin, it was thought possible that the Paradise nut might also yield a crystalline globulin.

Method.—A protein was isolated from Paradise nuts by extracting the defatted seed-meal with a saturated solution of sodium chloride and precipitating the protein with ammonium sulfate. The precipitate formed by saturating the protein solution with ammonium sulfate was redissolved in water and dialyzed against running distilled water at about 5°. Details of the method have been given elsewhere.[1] During dialysis crystals of globulin formed in the viscose bags. The crystals were thick, hexagonal plates. The edges of the upper and lower surfaces appeared to be beveled. Three of the angles of the sides were slightly more acute than those of the other three.

(1) Saunders, THIS JOURNAL, **53**, 696 (1931).

Experimental

Determinations of nitrogen distribution were made in the usual way. The results given below represent an average of several determinations.

	Protein, %		Total N, %
	Paradise-nut globulin	Excelsin[a]	Paradise-nut globulin
Amide N	1.42	1.48	9.12
Humin N	0.33	0.17	2.10
Total N in phosphotungstic acid ppt.	5.54	5.76	35.60
α-Amino N in phosphotungstic acid ppt.	2.06		13.28
Total N in filtrate (Kjeldahl)	8.46	10.97	53.51
α-Amino N in filtrate (Van Slyke)	8.43		53.44
Total N	15.75	18.30	
Histidine N (colorimetric)			5.23
Cystine N (gravimetric)			0.47

[a] Osborne and Harris as quoted by Plimmer, "Chemical Constitution of the Proteins," 2nd ed., Longmans, Green and Co., New York, 1924, p. 131.

Summary

A crystalline globulin has been isolated from the Paradise nut (*Lecythis zabucayo*).

The nitrogen distribution of this protein has been determined and found to be the same as excelsin except for mono-amino nitrogen.

CHICAGO, ILL. RECEIVED NOVEMBER 27 1936